α-AMINOADIPIC ACID AS A CONSTITUENT OF A CORN PROTEIN*

By EMANUEL WINDSOR

(From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California)

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In a study of transaminase systems Braunstein (1) learned that α-aminoadipic acid takes part in transamination. Both pigeon breast muscle mince and a purified glutamic transaminase extracted from it were active in transferring an amino group from alanine to α-ketoadipic acid. A plant aspartic transaminase was inactive with the same substrate.

Experiments by Borsook and Dubnoff (2) on the synthesis of arginine by rat kidney slices showed that, in addition to the activity of glutamic and aspartic acids in transferring amino groups to citrulline, lysine was active. They suggested at that time that lysine was converted to glutamic acid. Since aminoadipic acid was active in transamination, Braunstein suggested (3) that the lysine activity in arginine synthesis was due to its conversion into aminoadipic acid. Dubnoff and Borsook (4) repeated their previous experiments using aminoadipic acid as a substrate and found that it could aminate citrulline sufficiently well to explain the activity of lysine.

That one path of lysine degradation in guinea pig liver homogenate was through α-aminoadipic acid was proved by Borsook *et al.* (5) using radioactive L-lysine as a substrate and isolating radioactive α-aminoadipic acid from the reaction mixture. The aminoadipic acid was further degraded to α-ketoadipic acid and glutaric acid (6).

Geiger and Dunn (7) and Stevens and Ellman (8) independently found that aminoadipic acid was unable to replace lysine as an essential amino acid in rats and in two bacteria used in bioassays for lysine, *Leuconostoc mesenteroides* and *Streptococcus faecalis*.

The occurrence of free aminoadipic acid in biological material raises the possibility that this amino acid may be a protein constituent. In the research described herein evidence is presented that α-aminoadipic acid is part of a natural protein.

EXPERIMENTAL

1 part of protein was hydrolyzed with a minimum of 40 parts of 20 per cent hydrochloric acid, under a reflux on an oil bath for 20 hours. To

* Taken from a thesis in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

an aliquot of hydrolysate representing 2.5 mg. were added 9.3 γ of radioactive α-aminoadipic acid[1] (130 c.p.m.) and the excess hydrochloric acid was repeatedly evaporated *in vacuo*. Potato starch columns 30 cm. high were poured according to the directions of Stein and Moore (9); the labeled hydrolysate was taken up in a minimal quantity of 1:2:1 (*n*-butanol, *n*-propanol, 0.1 N HCl) solution (10) and then driven into the surface of the starch under pressure. A reservoir of 1:2:1 solution was attached to the top of the column and the column mounted on an automatic fraction collector adjusted to deliver 0.5 ml. fractions.

The odd numbered fractions were analyzed for amino nitrogen by the Moore and Stein quantitative ninhydrin method (11) and the even numbered fractions evaporated to dryness in stainless steel cups under heat lamps; the radioactivity was measured with a Geiger-Müller counter. It

TABLE I

Effluent Ratio on Starch Columns

Protein	Fraction No. at peak		Ratio
	Aminoadipic acid	Proline	
Casein	89	113	0.79
Gliadin	104	130	0.80
Zein	84	106	0.79
Cholera *Vibrio*	54	69	0.78
Average			0.79

was noticed that a constant ratio existed between the volumes of eluate at the aminoadipic acid peak (located by radioactivity) and the proline peak (yellow color with ninhydrin) (Table I). Although the volumes of eluate varied with the salt content of the hydrolysates and the water content of the starch, the ratio between the peaks remained constant. It was possible to conserve the limited supply of radioactive amino acid and rely on the position of the proline peak to locate the aminoadipic acid peak.

The proteins shown in Table II were tested for the presence of aminoadipic acid. The large majority of those examined showed no aminoadipic acid peak greater than could be accounted for by the added radioactive tracer. Fig. 1 is typical of the negative results given by these proteins, in this case a hydrolysate of crystalline zinc insulin to which 50 γ of proline had been added as a marker. The position of the aminoadipic acid locus was calculated from the proline peak. No trace of aminoadipic acid was

[1] Contributed through the generosity of Dr. H. Borsook and Dr. P. H. Lowy. The synthesis was previously described (6).

found. Similar results with cholera *Vibrio* have previously been published (12).

Analysis of Zein—When the amino acids in zein hydrolysates were thus

TABLE II

Proteins Examined for Aminoadipic Acid

		Source
Higher animals	Casein (vitamin-free)	Labco 9762
	Gelatin	Knox Sparkling
	Egg albumin	Merck and Company 31047
	Bovine hemoglobin	H. A. Itano*
	Human serum albumin	Fraction V, Harvard Medical School
	" γ-globulin	" II, " " "
	Cytochrome *c*	Nutritional Biochemicals Corporation
	Crystalline zinc insulin	Eli Lilly and Company 987267
	Pepsin, U. S. P.	City Chemical Corporation 7626
	Trypsin	
	Pancreatin	Eli Lilly and Company 2071-305175
	Erepsin	Nutritional Biochemicals Corporation
	Lipase (steapsin)	" " "
	Keratin	Merck and Company 43196
Higher plants	Gliadin	Bios Laboratories, Inc.
	Zein	Nutritional Biochemicals Corporation 1054
	α-Zein	Corn Products Refining Company*
	β-Zein	" " " "
	Corn glutelin	" " " "
	" pollen	H. J. Teas*
	Whole corn seed	" " "
	Lophophora williamsii	" " "
	Corn steep-water	Corn Products Refining Company 5203*
	Urease	Nutritional Biochemicals Corporation 1607
	Tobacco leaf cytoplasmic protein	S. Wildman*
	Almond meal	S. B. Penick and Company
	Papain, N. F.	Merck and Company 41678
Lower organisms	Ricin, c.p.	Eimer and Amend D29
	Cholera *Vibrio*	Eli Lilly and Company E-591*
	Escherichia coli	A. Siegal 7*
	Ulva species	H. J. Teas*
	Nereocystis luetkeana	" " "
	Tobacco mosaic virus	S. Wildman*
	Neurospora crassa	M. Fling*
	Taka-diastase (*Aspergillus oryzae*)	Parke, Davis and Company

* Generously contributed.

separated by starch columns, there was found 0.07 per cent aminoadipic acid (Fig. 2). More highly purified α-zein, soluble over a wide range of alcohol-water concentrations, and β-zein, soluble in 55 to 75 per cent alcohol, differing in their sulfur content, contained no aminoadipic acid.

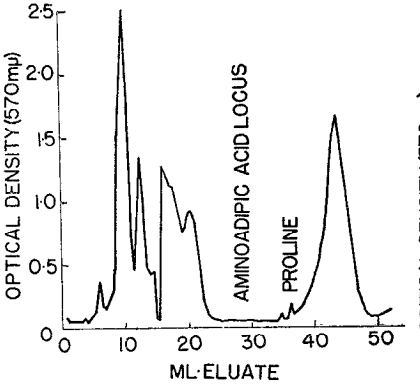

FIG. 1

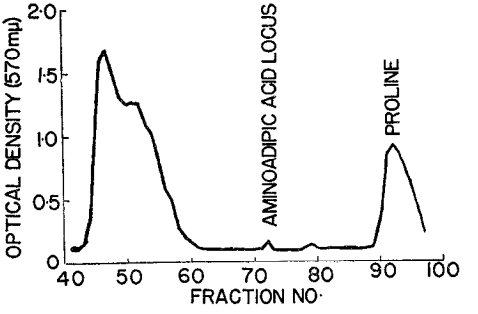

FIG. 2

FIG. 1. Starch chromatogram of crystalline zinc insulin hydrolysate. Column, 1 cm. in diameter. Hydrolysate, 2.5 mg. plus 50 γ of proline. Aminoadipic acid absent.

FIG. 2. Starch chromatogram of zein hydrolysate. Hydrolysate, 250 mg. Column, 8 cm. in diameter. Aminoadipic acid peak at Fraction 72; unknown ninhydrin-reacting substance at Fraction 79.

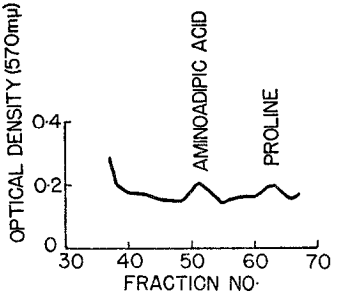

FIG. 3. Starch chromatogram of 28 day whole corn seed hydrolysate. Hydrolysate, 10 mg.; aminoadipic acid, 15 γ. Column, 1 cm. in diameter.

This suggested that the aminoadipic acid previously found in zein had been present as an impurity and that other protein fractions from corn might contain it in larger amounts. Accordingly, a sample of 28 day whole corn seed was hydrolyzed with hydrochloric acid and on analysis for aminoadipic acid was found to contain 0.15 per cent (Fig. 3).

Analysis of Corn Seed Proteins—A 3 gm. sample of 28 day whole corn seed ground to 40 mesh in a Wiley mill was extracted for 18 hours with

ether in a Soxhlet apparatus. The residue, insoluble in 10 per cent trichloroacetic acid, was dialyzed for 48 hours against four changes of distilled water until no more diffusible ninhydrin-reacting material came through the cellophane sac. The material in the dialysis sac was then fractionated by the Zeleny method (13), with the following modifications: each extraction was made with constant shaking for 3 hours and allowed

TABLE III

Fractionation of 3 Gm. Sample of 28 Day Ground Whole Corn Seed

Fraction No.	Soluble in	Total nitrogen	Protein*	Part of total protein	Part of total corn seed
		mg.	*mg.*	*per cent*	*per cent*
1	Water	11.4	71.0	24.0	2.4
2	N NaCl	2.7	17.0	5.8	0.6
3	80% ethanol	4.5	28.4	9.6	1.0
4	0.2% NaOH	10.4	65.3	22.1	2.2
5	Insoluble	18.2	113.7	38.5	3.8
Totals.....		47.2	295.4	100.0	10.0

* The factor 6.25 was used for the calculations.

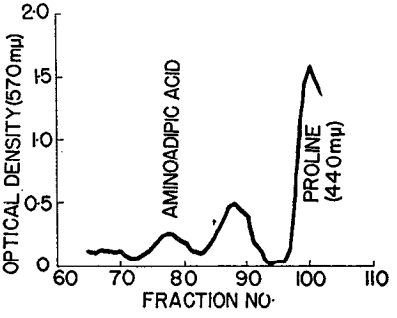

FIG. 4. Starch chromatogram of water-soluble corn seed protein. Concentrated eluate from 71 mg. of hydrolysate on medium column in aminoadipic acid region put on 25 mg. starch column. Aminoadipic acid, 43 γ, 0.06 per cent. Unknown substance at Fraction 89.

to stand 21 hours longer in the refrigerator, the insoluble residues were washed with 100 ml. of the extracting solutions, the 80 per cent alcohol was used cold, NaOH was used for the glutelin fraction instead of KOH. Each fraction was hydrolyzed with hydrochloric acid and nitrogen determinations were made by micro-Kjeldahl and direct nesslerization (Table III).

Each fraction was then analyzed for aminoadipic acid. The water-soluble non-dialyzable fraction contained 0.06 per cent (Fig. 4); the other fractions had no aminoadipic acid.

Fractionation of Corn Steep-Water Concentrate—Since corn steep-water concentrate, a by-product of the corn-milling industry, was available and a good source of the water-soluble portions of corn seed, a quantity was obtained[2] and analyzed for nitrogen, ash, and moisture (Table IV). The values obtained are in fairly good agreement with those of Cardinal and Hedrick (14).

A 100 mg. aliquot (wet basis) of the steep-water was hydrolyzed with hydrochloric acid, and 18.6 γ of radioactive aminoadipic acid (260 c.p.m.) were added as a marker and analyzed on a starch column. The radioactive peak in the eluate contained 132 γ of aminoadipic acid excess, or 0.13 per cent of the steep-water.

TABLE IV

Per Cent Composition of Steep-Water Concentrate

Specific gravity 1.25; pH 3.9.

Dry matter	53.8
Ash, dry basis	18.7
Total N, dry basis	8.3

TABLE V

Distribution of Aminoadipic Acid in Steep-Water Fractions

Fraction	Aminoadipic acid, per cent wet basis
Total	0.13
Free (dialysate)	0.08
Protein	0.05
Non-dialyzable, non-protein	<0.01

An unhydrolyzed 100 mg. aliquot was similarly analyzed and found to contain 72 γ excess or 0.07 per cent free aminoadipic acid.

The steep-water was fractionated to determine the distribution of aminoadipic acid (Table V).

Protein Fraction—70 gm. of steep-water were precipitated with trichloroacetic acid having a final concentration of 10 per cent; the precipitate was washed with water, acetone, and ether. The protein was hydrolyzed with hydrochloric acid and on analysis on a starch column was found to contain 0.05 per cent α-aminoadipic acid.

Non-Protein, Non-Dialyzable Fraction—The filtrate and washing from the protein precipitation were combined, the volume was reduced *in vacuo*, and the solution placed in a dialysis sac. Dialysis was carried out at 5°

[2] Generously contributed by A. L. Wilson of the Corn Products Refining Company, Argo, Illinois.

against four changes of distilled water containing 0.1 per cent phenol to prevent bacterial action. The contents of the sac were hydrolyzed with hydrochloric acid and analyzed after reduction in volume.

Free Amino Acid—The dialysate was reduced in volume, and an aliquot representing 200 mg. of steep-water was analyzed. Free α-aminoadipic acid amounted to 0.08 per cent.

Isolation of Aminoadipic Acid from Steep-Water Protein—A 2 kilo quantity of steep-water (wet weight) was diluted to 4 times its volume and strong sodium hydroxide solution added with stirring until the pH was raised to 5.0. The protein was precipitated by boiling the solution for 30 minutes with constant stirring to prevent bumping, and then allowed to cool to room temperature. The coagulated protein was filtered on a large Büchner funnel and washed with copious quantities of water, alcohol, and ether. The protein was dried to constant weight; yield, 73 gm., 3.7 per cent. Protein tests with the Millon and the Hopkins-Cole reagents were positive, as were the xanthoproteic reaction and the biuret test.

The protein was ground to a powder in a mortar, placed in a 3 liter round bottomed flask, and hydrolyzed with 1 liter of 20 per cent hydrochloric acid for 20 hours on an oil bath. Excess hydrochloric acid was repeatedly evaporated *in vacuo*. The dicarboxylic amino acids were precipitated twice as the calcium salts according to the method of Chibnall *et al.* (15). The calcium salts were dried and weighed; yield, 14.7 gm., 0.74 per cent.

The calcium salts were dissolved in water and calcium was removed with saturated oxalic acid. The filtrate and washings were reduced to 120 ml. A 15 ml. aliquot, to which had been added 93 γ of radioactive aminoadipic acid (1300 c.p.m.), was placed on a Dowex-50 column, prepared according to Stein and Moore (16), and eluted with 1.5 N HCl for 24 hours and 2.0 N HCl for 48 hours on an automatic fraction collector adjusted to deliver 10 ml. fractions. Aliquots of 0.5 ml. were taken from every alternate fraction and analyzed by the quantitative ninhydrin method to locate the peaks. Further 0.1 ml. aliquots of the fractions containing the ninhydrin-reacting material at the peaks were chromatographed on paper by the Williams and Kirby technique (17). Each peak was also tested for radioactivity. Thus it was learned that the aminoadipic acid was eluted immediately after glycine and before alanine, overlapping both of these amino acids (Fig. 5).

In this way the entire 120 ml., containing 650 γ of radioactive aminoadipic acid (9100 c.p.m.), were passed through eight Dowex-50 columns and all the fractions containing radioactive material were pooled. The solution was evaporated to dryness *in vacuo*, taken up in 1:2:1 solution, and placed on a 1 kilo starch column; 15 ml. fractions were collected on

the automatic fraction collector. Aliquots of 0.1 ml. were analyzed for amino nitrogen and radioactivity (Fig. 6). A single sharp peak was observed when the ninhydrin values and the radioactivity coincided. There was no overlapping with other ninhydrin peaks, and the other peaks contained no radioactivity. The α-aminoadipic acid in the peak equaled 17.5 mg.

Crystallization of Aminoadipic Acid—All the fractions in the peak were pooled and evaporated to dryness several times with additions of water to remove excess HCl. The residue was transferred to a beaker in 2 ml. of water and allowed to crystallize by evaporation in a desiccator. The

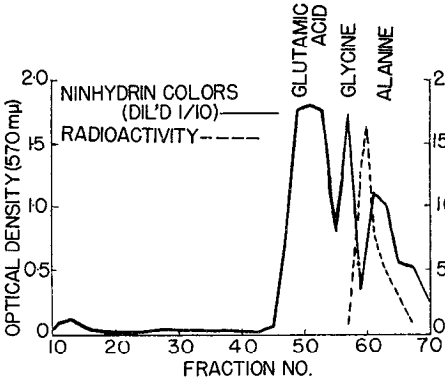

FIG. 5

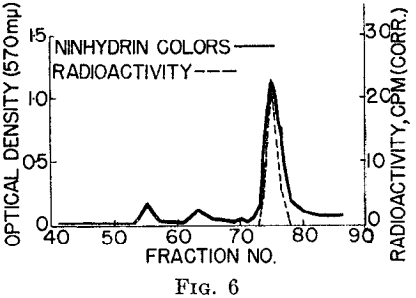

FIG. 6

FIG. 5. Dowex-50 chromatogram of hydrolyzed steep-water protein concentrate. 15 ml. aliquot of concentrate plus 93 γ of radioactive aminoadipic acid (1300 c.p.m.). Column, 85 cm. high and 2.3 cm. in diameter.

FIG. 6. Dowex-50 chromatogram of steep-water protein concentrate. 253 gm. of protein hydrolyzed, concentrated by Chibnall method (15), and passed through eight Dowex-50 columns. A 1 kilo starch column plus 650 γ of radioactive aminoadipic acid (9100 c.p.m.).

crystals which appeared were too hygroscopic to weigh; therefore it was necessary to make the free amino acid.

2 ml. of water were added, and the solution was titrated to pH 3.1 with 3 N ammonium hydroxide, 0.07 ml. being required. Electrodes and stirrer were washed with a small amount of water, and the washings added to the solution. To remove the yellow color 5 mg. of acid-washed norit were added to the solution and stirred for 1 hour. The norit was filtered and washed with a small amount of water.

The colorless aminoadipic acid solution was evaporated to 0.5 ml.; 3 ml. of 95 per cent alcohol were added and the precipitate which appeared was allowed to stand overnight in the refrigerator. The crystals were centrifuged, washed with small portions of 95 per cent alcohol and ether, and dried in air; weight 11.1 mg., specific activity 213.6 c.p.m.

5 mg. of the crystals were redissolved in a small amount of water, 5 mg. of norit again added, and the solution was heated, with occasional stirring, for 1 hour. The solution was filtered, filtrate and washing were reduced in volume to saturation, and 3 ml. of alcohol were added. The alcoholic solution was heated to boiling and allowed to cool in the refrigerator overnight. The crop of crystals was filtered, washed with small portions of alcohol and ether, and dried; weight 3.0 mg., specific activity 245 c.p.m. The melting point on an aluminum block was 206° with decomposition; mixed melting point with an authentic sample, 204–205° with decomposition.

Analysis—$C_6H_{11}O_4N$ (161.17). Calculated. C 44.72, H 6.88, N 8.70
Found. " 44.87, " 6.97, " 8.81

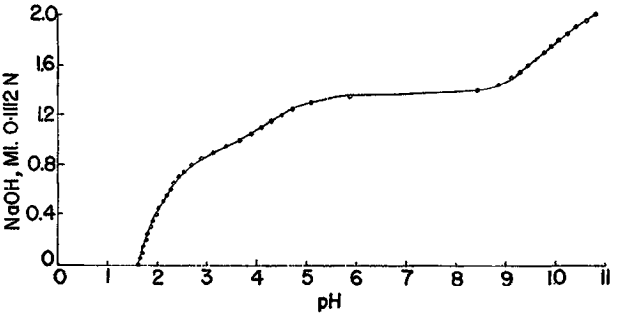

FIG. 7. Titration of aminoadipic acid with standard alkali. 0.05 mM of isolated aminoadipic acid dissolved in 1.3 ml. of water and titrated with 0.1112 N NaOH. pH determined by Beckman pH meter with glass and calomel electrodes.

Titration Curve—Isolated aminoadipic acid (0.05 mM) was dissolved in 1.3 ml. of water, titrated with 0.106 N'HCl to pH 1.5 with a Beckman pH meter, and then to pH 11.6 with 0.1112 N NaOH (Fig. 7).

The pK values were determined by the following methods: (*a*) The mid-point between inflection points on Fig. 7 was taken as the pK: pK_1, pK_2, and pK_3. (*b*) ΔpH was plotted against the constant increase of standard alkali, and the pK taken as the smallest value of ΔpH: pK_2 and pK_3. (*c*) pK_1 (Table VI) was calculated from an equation given by Schmidt (18): $pK_1 = pH - \log (C/(A - (H^+)) - 1)$, where A is concentration of acid and C is concentration of aminoadipic acid.

The ionization constants were pK_1 2.14, pK_2 4.21, pK_3 9.77, and pI 3.18.

Neurospora Growth—A *Neurospora crassa* mutant (strain 33933) grows on L-α-aminoadipic acid as well as L-lysine. However, growth on aminoadipic acid is inhibited by the presence of asparagine, while growth on lysine is stimulated by this amide (19). Flasks containing 20 ml. of minimal medium plus varying amounts of the isolated aminoadipic acid were inoculated with a suspension of conidia of mutant 33933. To one flask

was added asparagine. The mold was allowed to grow for 4 days at 25°. At the end of this time the mycelia were dried and weighed (Table VII).

Analysis of Taka-diastase—A 10 mg. sample of taka-diastase (an enzymatically active extract of *Aspergillus oryzae*) was hydrolyzed with HCl.

TABLE VI

pK_1 of Aminoadipic Acid

0.05 mM of aminoadipic acid in 1.3 ml. of water, titrated with 0.106 N HCl; pH values from Beckman pH meter with glass and calomel electrodes.

HCl	pH	pK_1	HCl	pH	pK_1	HCl	pH	pK_1
ml.			*ml.*			*ml.*		
0.12	2.70	2.09	0.32	2.20	2.15	0.54	1.89	2.20
0.14	2.62	2.09	0.34	2.18	2.19	0.58	1.83	2.15
0.15	2.60	2.11	0.36	2.15	2.20	0.62	1.77	2.06
0.16	2.58	2.13	0.38	2.11	2.18	0.70	1.70	2.14
0.18	2.52	2.14	0.44	2.03	2.22	0.74	1.68	2.09
0.20	2.44	2.10	0.48	1.97	2.23	0.78	1.65	2.07
0.24	2.36	2.14	0.50	1.92	2.15	0.90	1.58	2.07
0.30	2.23	2.14						
Average								2.14

TABLE VII

Growth of Neurospora Mutant 33933 on Isolated Aminoadipic Acid

20 ml. of minimal medium plus isolated aminoadipic acid autoclaved for 15 minutes at 20 pounds pressure, cooled to room temperature, and inoculated with 2 drops of a suspension of conidia of mutant 33933 in water. Mycelia allowed to grow for 96 hours at 25°, dried, and weighed.

Aminoadipic acid		Mycelium
mM	*mg.*	*mg.*
0	0	0
0.0025	0.40	0.6
0.005	0.80	4.2
0.01	1.61	13.9
0.015	2.41	27.8
0.02	3.22	41.0
0.005 + 0.02 mM asparagine		0

Radioactive aminoadipic acid (9.3 γ, 130 c.p.m.) was added as a marker and the hydrolysate was analyzed on a starch column. The ninhydrin-reacting material at the peak which coincided with the radioactivity contained 37 γ of aminoadipic acid in excess of the added marker, or 0.37 per cent. A larger sample was dialyzed for 48 hours against running water; non-dialyzable material was hydrolyzed with HCl and analyzed on a starch column. No trace of aminoadipic acid was found in this fraction.

DISCUSSION

The discovery of α-aminoadipic acid as a constituent of a protein in corn steep-water by means of starch chromatography and confirmation of its presence there by isolation of the amino acid are a tribute to the precision of this chromatographic method. While the water-soluble protein in corn seed may not be the same as the protein in steep-water, the chromatographic data show that α-aminoadipic acid is also present in the natural seed protein. The nature of the linkage is unknown. Adsorption of free amino acid can be ruled out by the prolonged dialysis to which the protein was subjected. Secondary changes of lysine in protein as an explanation, while a possibility, are not likely, since all the proteins examined were treated to the same conditions and only the corn proteins, in seeds and in steep-water, showed the presence of α-aminoadipic acid.

Evidence was seen of an unknown ninhydrin-reacting substance (Fig. 4) in water-soluble corn seed protein and in the corn steep-water hydrolysate. The mobility of this substance on a starch column differs from anything previously described.

The presence of free α-aminoadipic acid in the extract of *A. oryzae* (taka-diastase) suggests that further investigation of the mold should be undertaken.

SUMMARY

Thirty-five proteins or protein-containing materials were analyzed for α-aminoadipic acid by chromatography on starch columns. Of these materials corn seed, corn steep-water, and taka-diastase (*Aspergillus oryzae*) were shown to contain free α-aminoadipic acid.

As a protein constituent aminoadipic acid was found in a water-soluble corn seed protein and was shown to be present in corn steep-water protein by its isolation from a hydrolysate. Its identity with the synthetic amino acid was proved by elementary analysis, mixed melting point, chromatographic behavior on both starch and Dowex-50 columns, use as a growth substance for a *Neurospora crassa* mutant which requires it for growth, and inhibition of this growth by a specific inhibitor.

The ionization constants for the isolated aminoadipic acid in water were determined.

An unknown ninhydrin-reacting substance was found in the water-soluble corn seed protein and in corn steep-water.

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